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13. ABSTRACT ***(Maximum 200 Words)***

Activation of tyrosine kinases plays a key role in cell proliferation, and ErbB receptor tyrosine kinases are specifically implicated in breast cancer. Biochemical studies have recently identified the proto-oncogene product Cbl as a negative regulator of EGF receptor and ErbB2. The Cbl-dependent negative regulation of ErbB receptors was associated with their ubiquitin modification and downregulation from the cell surface. Based on these observations, this proposal is investigating the role of Cbl-mediated ubiquitination as a signal for targeting activated ErbB receptors to lysosomes where they undergo degradation. The work reported here has focused on characterization of a series of mutant forms of Cbl that are unable to mediate ubiquitination of EGFR. The analyses of these mutants support the role of ubiquitination in Cbl-mediated cell surface regulation of EGFR. Further studies will establish the intracellular targeting of downregulated ErbB receptors to lysosomes, and utilize a number of biological systems to directly explore if ubiquitination is an essential mechanism for sorting ErbB receptors to lysosomes. The present studies, thus, aim to define novel strategies to downregulate proliferative signals in breast cancer cells.

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A Novel Pathway to Down-Regulate ErbB Signaling in Mammary Epithelial Cells

Introduction:

The experiments proposed in the application submitted for funding were designed to test a unique hypothesis that the proto-oncogene product Cbl down-regulates ErbB signals in mammary epithelial cells by controlling a ligand-induced ubiquitination, internalization and degradation pathway. Genetic studies, initially in C. elegans and Drosophila systems and recently using mouse knock-outs, as well as extensive biochemical studies have established Cbl as a negative regulator of tyrosine kinases. At the time of this application, the TKB domain of Cbl had been shown to be crucial for Cbl function, which formed the basis for the use of TKB domain mutants in our studies. Subsequent studies in our laboratory demonstrated that the RING finger domain of Cbl was crucial for negative regulation of EGFR and Syk tyrosine kinases. Furthermore, the TKB and RING finger domain (together with short conserved regions surrounding the RING finger domain) were sufficient for Cbl-induced negative regulation of EGFR and Syk, as well as for Cbl-mediated ubiquitination of EGFR. Given the ability of Cbl to support ubiquitination of target proteins in vitro, the TKB and RING finger domains of Cbl together define a novel ubiquitin ligase module that specifically targets activated tyrosine kinases like ErbB receptors for ubiquitination. Understanding the mechanisms of this novel biochemical machinery and how ubiquitin modification mediates negative regulation of ErbB receptors is likely to provide crucial insights of biological and medical significance.

Modifications to Statement of Work:

In order to test the hypothesis proposed in the application, we proposed to express GFP-tagged Cbl or its mutants in ErbB receptor-expressing cells, analyze their association with ErbB receptors, assess their effects on ligand-induced ubiquitination, internalization and degradation of the ErbB receptors, and determine their effects on EGF-dependent mitogenic responses. Given the ongoing studies in our laboratory (Lill et al., JBC 275:367-377, 2000), which indicated a crucial role for the RING finger domain of Cbl and the adjacent regions in mediating ubiquitination of EGF receptor, we considered it important to establish a well-characterized set of Cbl mutants, which could be expressed as stable proteins in cells and whose effects on ErbB ubiquitination were well-established. For this purpose, we have carried out extensive mutagenesis analyses and have identified a number of new mutants that now allow us to undertake our proposed goal. We have also set up an in vitro ubiquitination assay and modified our in vivo ubiquitination assay to rapidly correlate the specific mutations in Cbl to their effects on the ubiquitin ligase function of Cbl. In addition, we have set up a genetic system of cells with a temperature-sensitive ubiquitin-conjugating enzyme (Strous et al., EMBO J. 15:3806, 1996) to allow us to establish the cause and effect relationship between Cbl-mediated ubiquitination and down-regulation of ErbB receptors.

The modified statement of work for the next year is:

1. Examine the site of action of Cbl-dependent ubiquitination in EGFR down-regulation

   A. Assess if Cbl-mediated ubiquitination is a pre-internalization or post-internalization process.
   B. Determine the identity of vesicular compartments in which Cbl co-localizes with the EGFR
   C. Assess the nature of vesicular compartments in which the dominant-negative Cbl mutants co-localize with the EGFR.
   D. Assess the effects of conditional inactivation of ubiquitination machinery on Cbl-dependent traffic of the EGFR.

2. Generate mammary epithelial cells expressing defined Cbl mutants to test the role of Cbl in EGFR down-regulation

   A. Introduce GFP constructs encoding mutant Cbl proteins into mammary epithelial cells.
   B. Demonstrate the expression of introduced Cbl proteins and their phosphorylation and association with EGFR upon ligand stimulation.
   C. Determine the effects of introduced Cbl proteins on mitogenic response to EGF.

Body:

Studies carried out in the current reporting period helped further the training of the trainee, and resulted in the generation of a number of Cbl mutants as well as the establishment of technical procedures that will facilitate the achievement of our goals.

A naturally occurring mutation that converts the negative regulatory protein Cbl into a potent oncogene corresponds to deletion of a17-amino acid region (residues 366-382) adjacent to N-terminal boundary of the RING finger domain. Modeling of this region (the linker region) predicted a helical region (data not shown) and this has been confirmed by a recent crystal structure (Zheng et al., Cell 102:533, 2000). The latter study also showed intimate contacts of the liker helix with both the tyrosine kinase-binding (TKB) domain of Cbl and the RING finger-associated ubiquitin-conjugating enzyme (UBC). Thus, the linker region appeared likely to be critical for Cbl ubiquitin-ligase function towards tyrosine kinase targets. To test if this was the case, we generated a number of mutants that carry substitution or deletion mutations within this region and tested these for their ability to facilitate the ligand-induced ubiquitination and cell surface down-regulation of the EGFR.

The expression constructs encoding the green fluorescent protein (GFP)-tagged Cbl proteins were generated by a two-step procedure as follows. First, mutations were introduced into the wild-type Cbl coding sequence by oligonucleotide-directed

mutagenesis using pAlterMAX-HA-Cbl and the Altered Sites II mutagenesis system (Promega, Madison, WI; see Appendix Manuscript, Table I). Mutant coding sequences were transferred as EcoRI-XbaI fragments from mutagenized pAlterMAX-HA-Cbl constructs into EcoRI-XbaI-digested pCDNA-3-GFP-Cbl. For generation of the deletion mutant constructs pCDNA-3-GFP-Cbl-70Z, pCDNA-3-GFP-Cbl-Δ368, and pCDNA-3-GFP-Cbl-Δ371, mutant Cbl coding sequences were amplified from the expression constructs pSRαneo-Cbl-70Z, pJZenNeo-Cbl-del368 and pJZenNeo-Cbl-del371, respectively. The pJZenNeo expression constructs were the kind gifts of Wallace Y. Langdon (University of Western Australia, Nedlands, Western Australia). Amplification primers were 5'-CCC-GGA-TCC-GCC-GGC-AAC-GTG-AAG-AAG-AGC-3' and 5'-GGG-CTC-GAG-TTA-CTA-GGT-AGC-TAC-ATG-GGC-AGG-A-3'. BamHI-XhoI-digested amplification products were ligated to BamHI-XhoI-digested pCDNA-3-GFP-Cbl. All mutations introduced by oligonucleotide-directed mutagenesis were confirmed by DNA sequencing. All Cbl-coding sequences derived through PCR amplification were sequenced in their entirety.

To assess the impact of liker mutations on the ability of the Cbl protein to down-regulate the EGFR, we utilized a novel two-color fluorescence activated cell sorter (FACS)-based quantitative assay of the ligand-dependent EGF-R down-regulation recently developed in the laboratory (Lill et al., JBC 275:367-377, 2000). In brief, HEK 293 cells expressing low levels of endogenous EGF-R are transiently transfected with expression constructs encoding EGF-R and wild-type or mutant GFP-tagged Cbl proteins. Following incubation with EGF for times ranging from 0 to 40 min, matched transfection cultures are harvested and processed for surface EGF-R immunostaining using a secondary antibody conjugated to phycoerythrin (red fluorescence). Cell surface EGF-R levels (red fluorescence) of transfected cell populations (green fluorescence due to GFP) are expressed relative to the surface receptor levels of un-stimulated cells.

A time course study of ligand-dependent EGF-R down-regulation by Cbl deletion mutants was performed. As expected from previous studies, receptor down-regulation was enhanced upon over-expression of wild-type Cbl (Appendix Manuscript, Fig.2, compare squares and diamonds) and suppressed by the oncogenic deletion mutant Cbl-70Z (Appendix Manuscript, Fig.2, compare circles and diamonds). The Cbl-70Z protein lacks amino acids 366-382 and functions as a dominant negative Cbl protein in assays of EGF-R down-regulation and ubiquitination. The activity of Cbl-70Z was similar to that reported for the transforming N-terminal truncation mutant Cbl-N, which comprises amino acids 1-357. When Cbl linker region mutants, Δ368 and Δ371, were over-expressed (Appendix Manuscript, Fig.2, triangles and –X-, respectively), they functioned similar to the dominant-negative mutants Cbl-70Z and Cbl-N.

The Δ368 and Δ371 Cbl mutants also were evaluated for their ability to enhance EGF-R ubiquitination. HEK 293 cells were transiently transfected using EGF-R and GFP-Cbl expression constructs as outlined above. The cells were harvested without or following EGF stimulation of matched transfection cultures. EGF-R ubiquitination was assessed by combined immunoprecipitation and immunoblotting (Fig.3). Whereas the over-expression of wild-type Cbl enhanced the ligand-dependent EGF-R ubiquitination (Appendix Manuscript, Fig. 3, compare lanes 2 and 4), the transforming deletion mutant proteins Cbl-70Z, Δ368 and Δ371 suppressed the ubiquitination (Fig. 3, compare lane 2

with lanes 6, 12, and 14, respectively). Each deletion mutant protein was able to associate with EGF-R (Bowtell and Langdon, 1995 and data not shown), yet failed to enhance the receptor down-regulation and ubiquitination. Thus, the transformation potential of Cbl linker region deletion mutants correlates with their dominant negative behavior in these assays.

The contribution of Cbl amino acids Y368 and Y371 to EGF-R regulation was further investigated using amino acid substitution mutants targeting these residues. A panel of mutants was generated for the individual substitution of phenylalanine (F), glutamic acid (E), or alanine (A) for each tyrosine (Appendix Manuscript, Table I). Substitutions were chosen for one of the following reasons: 1) to conserve structure while preventing phosphorylation of the target residue (Y-to-F); 2) to alter structure while conserving the charge that would result from tyrosine phosphorylation (Y-to-E); 3) to alter both structure and potential charge (Y-to-A).

The activity of the substitution mutants was evaluated in the EGF-R down-regulation assay. All three Y371 substitution mutants functioned as dominant-negative regulators of surface EGF-R down-regulation (Fig.4, open symbols/solid lines), thereby mimicking the activity of the dominant-negative Δ371 Cbl mutant (Fig.2, -X-). However, Y368 substitution mutants were as active as wild-type Cbl in enhancing EGF-R down-regulation (Fig.4, compare squares with open symbols/dashed lines). These results contrasted with the dominant-negative function of mutant Δ368 in this assay (Fig.2, triangles).

The impact of the Y368 and Y371 substitution mutants on EGF-R ubiquitination was determined. Each Cbl mutant was able to associate with the EGF-R (data not shown). While the Y368 substitution mutants enhanced EGF-R ubiquitination to the same degree as the wild-type Cbl (Fig.5 middle panel, compare lanes 4, 6, 8, and 10 with lane 2), the Y371 substitution mutants effected no enhancement (Fig.5 middle panel, compare lanes 12, 14, and 16 with lane 2). Control immunoblots of cell lysates confirmed that EGF-R (Fig.5, top panel) and wild-type and mutant Cbl proteins were expressed at equivalent levels in all transfections, with the exception of the Y371A mutant protein (Fig.5, bottom panel). Immunofluorescence microscopy of cells expressing the Y371A mutant protein revealed accumulation of GFP-tagged Cbl in cytoplasmic aggregates, rather than the cytosolic distribution observed for wild-type or other mutant Cbl proteins (not shown). Aberrant intracellular localization correlated with an apparent reduction of protein solubility in the lysis buffer used in the combined immunoprecipitation and immunoblotting analysis (Fig.5 bottom panel, lanes 15 and 16).

Taken together, our results indicate that a tyrosine at position 371 of Cbl is indispensable for normal regulation of the EGF-R, while the identity of the residue at position 368 is not critical. Instead, amino acid 368 serves as a critical spacer residue within the Cbl linker region.

In addition to Y368 and Y371, three other linker region amino acids (T364, S376, and T377) were targeted as these represent potential sites of ligand-dependent phosphorylation. Using expression constructs encoding singly substituted Cbl proteins (see Table I), we assessed the contribution of these amino acids to Cbl-mediated enhancement of EGF-R down-regulation and ubiquitination. Substitutions were designed

to effect either of two changes: 1) to mimic the charge that would be induced upon phosphorylation of the residue without conserving amino acid structure (S- or T-to-D); or 2) to conserve neither amino acid structure nor potential charge (S- or T-to-A). The results of the EGF-R down-regulation assays using the linker serine and threonine substitution mutants are shown in Figure 6 of the Appendix Manuscript. Mutations at position 364 gave varied results. While the T364D mutant functioned like wild-type Cbl in enhancing EGF-R down-regulation (Fig.6A, -X-), Cbl T364A was compromised for this activity (Appendix Manuscript, Fig.6A, triangles). In several ways, the GFP-T364A mutant resembled the Y371A mutant: it localized to cytoplasmic aggregates (data not shown) and was poorly soluble in the lysis buffer used to prepare cell lysates (Appendix Manuscript, Fig.7, bottom panel, lanes 4 and 12). However, the dominant-negative activity of the Y371A mutant was not reflected by the T364A mutant, which slightly enhanced EGF-R down-regulation in this assay. Substitution of alanine for serine at position 376 had some impact in the down-regulation assay. The mutant enhanced EGF-R down-regulation, but to a lesser degree than did wild-type Cbl (Fig.6B, compare triangles and squares). The Cbl T377D mutant was functionally null for enhancement of EGF-R down-regulation (Fig.6C, -X-). However, the T377A mutant enhanced down-regulation to near wild-type levels (Fig.6C, triangles).

These data show that at least one amino acid substitution at any of the three linker region serine or threonine residues can reduce or abrogate Cbl-mediated enhancement of EGF-R down-regulation. To confirm that these mutations similarly compromise EGF-R ubiquitination, we tested the mutants in the combined immunoprecipitation and immunoblotting procedure (Fig.7). All Cbl mutants that enhanced EGF-R down-regulation also enhanced ligand-dependent receptor ubiquitination (Fig.7, middle panel, compare lanes 10, 11, 12, and 14 with lane 8). These included mutants T364D, T364A, S376A, and T377A. The T377D mutant, unable to enhance EGF-R down-regulation, was severely compromised in its ability to enhance receptor ubiquitination (Fig.7, middle panel, compare lane 13 with lane 8).

In summary, results from the linker region mutant analyses in the 293 HEK system suggest that the Cbl activities that mediate the enhanced ligand-dependent EGF-R down-regulation and enhanced ligand-dependent receptor ubiquitination are linker-dependent and genetically inseparable. Notably, the phenotype of the Y371F Cbl mutant indicates that loss of the EGFR down-regulation and ubiquitination function is not sufficient to convert Cbl into an oncogene since Y371F, unlike Y371Δ mutant, is not oncogenic. Recent results from another laboratory using Cbl RING finger mutants (Thien et al, Mol. Cell 7:355, 2001) support this conclusion.

Thus, a series of well-characterized GFP-tagged Cbl mutants is now available for further studies in mammary epithelial cells and other cell systems in order to delineate the mechanisms whereby Cbl-mediated ubiquitination regulates the down-regulation of the EGFR.

Key Research Accomplishments:

- Generated a panel of new HA-tagged Cbl mutants carrying mutations in the "linker" region of Cbl located between the TKB and RING finger domains.

- Generated a panel of GFP-tagged Cbl mutants.

- Identified the linker region as a new motif that is essential for Cbl ubiquitin ligase activity towards EGFR and for cell surface EGFR down-regulation in HEK293 system.

Reportable Outcomes:

Publications:

- Nancy L. Lill, **Karen P. Mullane-Robinson**, Satoshi Ota, Navin L. Rao, Nichole Meissner-Lula, and Hamid Band. Mutational analysis of the linker region of Cbl reveals its critical role in the ubiquitination and down-regulation of the epidermal growth factor receptor. Manuscript in preparation.

Reagents:

- Generated a panel of new HA-tagged Cbl mutants carrying mutations in the "linker" region of Cbl located between the TKB and RING finger domains.

- Generated a panel of GFP-tagged Cbl mutants carrying mutations in the "linker" region of Cbl located between the TKB and RING finger domains.

Funding applied for based on this work:

The work carried out under this award will be part of the background and preliminary studies for an NIH RO1 renewal application (Regulating Tyrosine Kinase Signals in Breast Cancer) that is being submitted by the applicant's mentor, Dr. Band, later this year.

Manuscripts included:

- Nancy L. Lill, **Karen P. Mullane-Robinson**, Satoshi Ota, Navin L. Rao, Nichole Meissner-Lula, and Hamid Band. Mutational analysis of the linker region of Cbl reveals its critical role in the ubiquitination and down-regulation of the epidermal growth factor receptor. Manuscript in preparation.

Conclusions:

In conclusion, our results implicate the linker region of Cbl, located between its TKB and RING finger domains, as a crucial element for Cbl-mediated ubiquitination and down-regulation of the EGF receptor. The linker region is the most highly conserved motif among Cbl family proteins and is highly conserved during evolution. Thus, the findings presented here are likely to be of general significance for all Cbl family proteins. Importantly, the reagents developed here now allow us direct analyses of the mechanism of EGFR down-regulation by Cbl-mediated ubiquitination. Furthermore, we are now uniquely poised to undertake the proposed analyses to elucidate the role of Cbl in regulating the EGFR signaling in mammary epithelial cells.

Mutational analysis of the linker region of Cbl reveals its critical role in the ubiquitination and down-regulation of the epidermal growth factor receptor

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Running Title: Cbl linker region regulates EGF receptor down-regulation

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Summary

The proto-oncoprotein Cbl suppresses signaling by receptor and non-receptor tyrosine kinases. Recent reports indicate that Cbl negatively regulates epidermal growth factor receptor (EGF-R) signaling by enhancing receptor ubiquitination, down-regulation from the cell surface, and degradation. We have shown that the N-terminal 440 amino acids of Cbl are sufficient for EGF-R regulation. These Cbl sequences encompass the N-terminal tyrosine kinase-binding domain (residues 1-357), a linker region (residues 358-380), the RING finger domain (residues 381-419), and a short stretch of C-terminal amino acids (residues 420-440). On examination of these domains for motifs likely to regulate ligand-dependent EGF-R ubiquitination and degradation, we identified the Cbl linker region as a potential PEST-like sequence. PEST-like sequences have been shown to target proteins for internalization or degradation by the 26S proteasome. Because negative regulation of the EGF-R by Cbl is ligand-dependent, we assessed the impact of mutating five Cbl linker region residues that might undergo inducible phosphorylation upon receptor activation. Individual mutations at each of the five sites (T364; Y368; Y371; S376; T377) compromised Cbl-mediated EGF-R ubiquitination and down-regulation. Our results suggest that the Cbl linker region is a functional and structural domain critical for Cbl-mediated negative regulation of the EGF-R.

Introduction

The proto-oncoprotein Cbl is a regulator of numerous receptor and non-receptor protein tyrosine kinases (reviewed in 1-7). Cbl inducibly associates with activated kinases, undergoes tyrosine phosphorylation, and serves as a substrate for the recruitment of additional signaling proteins.

In addition to its role as an adapter protein for formation of multimolecular signaling complexes, Cbl functions as a suppressor of protein tyrosine kinase signaling. Evidence for Cbl-mediated negative regulation of receptor tyrosine kinases first emerged from *in vivo* studies of *Caenorhabditis elegans*. The *C. elegans* Cbl homologue, SLI-1, negatively regulates signaling through the epidermal growth factor receptor (EGF-R)[1] homologue LET-23 (8). Cbl-mediated suppression of EGF-R-dependent R7 photoreceptor neuron development in *Drosophila* and mammary fat pad development in mice confirmed that Cbl critically regulates EGF-R signaling pathways *in vivo* (9-11).

Recent studies have shown that expression of Cbl in cultured mammalian cells enhances activation-dependent ubiquitination, down-regulation, and/or degradation of multiple receptor tyrosine kinases (12-15). We recently reported that Cbl amino acids 1-440 are sufficient to effect enhanced ubiquitination and down-regulation of the EGF-R (16). This region encompasses the evolutionarily conserved Cbl sequences 1-436 (9, 17) and comprises several structural domains (Fig.1). These include a tyrosine kinase-binding (TKB) domain (amino acids 1-357), a linker region (amino acids 358-380), a zinc-coordinating RING finger domain (amino acids 381-419), and a short stretch of C-terminal residues (amino acids 420-440). Structure-function studies have revealed important roles for the Cbl TKB (16) and RING finger (18-20) domains in

enhancing ubiquitination and down-regulation of the EGF-R. Functional activities of the Cbl linker region and C-terminal amino acids 420-440 are less well-defined.

To understand how Cbl amino acids 1-440 suppress EGF-R signaling, we examined this sequence for motifs likely to regulate ligand-dependent EGF-R ubiquitination and degradation. We have identified the Cbl linker region as a PEST-like sequence. PEST sequences are protein regions enriched in proline (P), aspartic and/or glutamic acid (E), serine (S) and/or threonine (T) residues, and flanked by basic amino acids (21;22). Similar to PEST sequences, PEST-like sequences are enriched in acidic and hydroxylated amino acids, but may lack proline residues (23). PEST and PEST-like sequences have been shown to target proteins for internalization or degradation by the 26S proteasome (23-32). Both of these activities may be induced upon phosphorylation of amino acids within the PEST or PEST-like sequences (23, 24, 26, 27, 29, 33-37).

Because Cbl-mediated enhancement of EGF-R ubiquitination, down-regulation, and degradation is ligand-dependent, we assessed the impact of mutating the five Cbl linker region residues that might undergo inducible phosphorylation upon receptor activation. We report that specific mutations at each of these sites (T364; Y368; Y371; S376; T377) compromise or abrogate Cbl-mediated enhancement of EGF-R ubiquitination and down-regulation. Furthermore, the structure-function results from the mutational analysis of residue Y368 suggest that the Cbl linker region comprises a structural motif that regulates protein tyrosine kinase signaling.

Experimental Procedures

Plasmids-- The plasmids pAlterMAX-HA-Cbl, pAlterMAX-EGF-R, pCDNA-3-GFP, pCDNA-3-GFP-Cbl, and pCDNA-3-GFP-Cbl-N have been described (16). Constructs encoding green fluorescent protein (GFP) fused to Cbl linker region mutant proteins substituted at a single amino acid were generated by a two-step procedure. First, mutations were introduced into the wild-type Cbl coding sequence by oligonucleotide-directed mutagenesis using pAlterMAX-HA-Cbl and the Altered Sites II mutagenesis system (Promega, Madison, WI; see Table I). Mutant coding sequences were transferred as EcoRI-XbaI fragments from mutagenized pAlterMAX-HA-Cbl constructs into EcoRI-XbaI-digested pCDNA-3-GFP-Cbl. For generation of the deletion mutant constructs pCDNA-3-GFP-Cbl-70Z, pCDNA-3-GFP-Cbl-Δ368, and pCDNA-3-GFP-Cbl-Δ371, mutant Cbl coding sequences were amplified from the expression constructs pSRαneo-Cbl-70Z, pJZenNeo-Cbl-del368 and pJZenNeo-Cbl-del371, respectively. The pJZenNeo expression constructs were the kind gifts of Wallace Y. Langdon (University of Western Australia, Nedlands, Western Australia). Amplification primers were 5'-CCC-GGA-TCC-GCC-GGC-AAC-GTG-AAG-AAG-AGC-3' and 5'-GGG-CTC-GAG-TTA-CTA-GGT-AGC-TAC-ATG-GGC-AGG-A-3'. BamHI-XhoI-digested amplification products were ligated to BamHI-XhoI-digested pCDNA-3-GFP-Cbl. All mutations introduced by oligonucleotide-directed mutagenesis were confirmed by DNA sequencing. All Cbl-coding sequences derived through PCR amplification were sequenced in their entirety.

Antibodies-- The anti-EGF-R murine monoclonal (EGFR 528; sc-120, IgG2a), anti-EGF-R rabbit polyclonal (EGFR 1005; sc-03), anti-Cbl rabbit polyclonal (C-15; sc-170), and anti-Syk

murine monoclonal (Syk 4D10; sc-1240, IgG2a) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-GFP rabbit polyclonal antibody RDI-GRNFPabR was obtained from Research Diagnostics, Inc. (Flanders, NJ). Anti-class I HLA monoclonal antibody W6/32 (IgG2a; Barnstable et al., 1978) was provided by Christopher Roy and Samuel Behar (Brigham and Women's Hospital, Boston, MA). Rabbit polyclonal anti-ubiquitin antibody NCL-UBIQ (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK) was purchased from Vector Laboratories, Inc. (Burlingame, CA). The secondary antibody used for live cell immunostaining, R-phycoerythrin-conjugated AffiniPure F(ab')$_2$ fragment of goat anti-mouse IgG (H+L), was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Cells-- Human embryonic kidney (HEK) epithelial cell line 293 (38) was obtained from the American Type Culture Collection and maintained in supplemented Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Gaithersburg, MD) containing 10% fetal bovine serum (FBS) as described (Lupher et al., 1998).

Transient Transfection, EGF Stimulation, and Preparation of Lysates of Mammalian Cells-- These procedures were performed as recently described (16). In brief, transfections were performed using the calcium phosphate method (39, 40). The amounts of input DNA are indicated in the appropriate figure legends. Cultures were harvested at 36-48 h following the addition of DNA precipitates. Cells were serum-starved for 4-6 hr prior to harvest without or following incubation with purified murine EGF (Sigma) at 100 ng/ml for the times indicated. Cell lysates were prepared using Triton X-100 lysis buffer [50 mM Tris, (pH 7.5), 150 mM

sodium chloride, 0.5% Triton X-100 (Fluka), 1 mM phenylmethylsulfonyl fluoride, 0.07 trypsin inhibitor units aprotinin/ml, and 1 μg/ml each of leupeptin, pepstatin, antipain, and chymostatin]. The Bradford protein assay (Bio-Rad Laboratories, Hercules, CA) was used to determine lysate protein concentration.

Immunoprecipitation and Immunoblotting-- The procedures followed for protein immunoprecipitation and immunoblotting were described previously (16, 41). The amounts of lysate protein and antibodies used are indicated in the relevant figure legends. Where indicated, the transfer membranes were stripped and reprobed.

Live Cell Immunostaining and Flow Cytometry -- The procedure used was described in detail previously (16). All samples of transfected HEK 293 cells were immunostained in triplicate using anti-Syk (negative control), anti-EGF-R, and anti-class I (positive control) antibodies. Following immunostaining, fixed cells were analyzed for GFP- (green) and EGF-R- (red) specific fluorescence. Flow cytometry, data collection, and analysis were performed using a FACSort machine and CellQuest software (Becton Dickinson, Franklin Lakes, NJ). For each sample, 5000 green fluorescent cells (i.e., expressing GFP-Cbl proteins; typically present at 15-30% of the population) were analyzed. In each case, the mean fluorescence intensity (MFI) value for the negative control antibody was subtracted from the MFI value for the anti-EGF-R antibody to yield the specific MFI of EGF-R staining. All down-regulation assays were performed as three separate experiments. Although the relative positions of the Cbl mutant curves were consistent over three experiments when compared to the internal control curves (GFP alone, GFP-Cbl, and GFP-70Z), variation in the spread of the control curves occurred from

experiment to experiment. For this reason, representative curves from a single experiment are shown.

Results

Deletion of Cbl Amino Acid Y368 or Y371 Abrogates Cbl-mediated Enhancement of EGF-R Down-regulation and Ubiquitination-- Cbl-mediated enhancement of EGF-R down-regulation, ubiquitination, and degradation is ligand-dependent. This suggests that inducible post-translational modification of Cbl may be required for receptor regulation. We previously demonstrated that the N-terminal 440 amino acids of Cbl are sufficient to effect enhanced EGF-R down-regulation and ubiquitination (16). The linker region of Cbl (amino acids 358-380) is encompassed by these sequences (Fig.1), and mutations affecting the linker region can convert Cbl into an oncoprotein (42). Specifically, deletion of Cbl linker region residue Y368 or Y371 confers transforming potential on Cbl (42). Because these sites are contained within the biologically active N-terminus of Cbl, could potentially undergo activation-dependent phosphorylation, and are crucial for the integrity of normal Cbl function, we evaluated their contribution to Cbl-mediated down-regulation and ubiquitination of the EGF-R.

We recently described a novel assay to quantify ligand-dependent EGF-R down-regulation from the cell surface (16). In brief, HEK 293 cells expressing low levels of endogenous EGF-R are transiently transfected with expression constructs encoding EGF-R and wild-type or mutant GFP-tagged Cbl proteins. Following incubation with EGF for times ranging from 0 to 40 min, matched transfection cultures are harvested and processed for surface EGF-R immunostaining. Cell surface EGF-R levels (red fluorescence) of transfected cell populations (green fluorescence) are expressed relative to the surface receptor levels of unstimulated cells.

A timecourse study of ligand-dependent EGF-R down-regulation by Cbl deletion mutants was performed. As expected (13, 16, 20), receptor down-regulation was enhanced upon overexpression of wild-type Cbl (Fig.2, compare squares and diamonds) and suppressed by the

oncogenic deletion mutant Cbl-70Z (Fig.2, compare circles and diamonds). The Cbl-70Z protein lacks amino acids 366-382 and functions as a dominant negative Cbl protein in assays of EGF-R down-regulation and ubiquitination (20). The activity of Cbl-70Z was similar to that reported for the transforming N-terminal truncation mutant Cbl-N, which comprises amino acids 1-357 (13, 16). When Cbl linker region mutants Δ368 and Δ371 were overexpressed (Fig.2, triangles and –X-, respectively), they functioned like the dominant negative mutants Cbl-70Z and Cbl-N.

The Δ368 and Δ371 Cbl mutants also were evaluated for their ability to enhance EGF-R ubiquitination. HEK 293 cells were transiently transfected using EGF-R and GFP-Cbl expression constructs as outlined above. The cells were harvested without or following EGF stimulation of matched transfection cultures. EGF-R ubiquitination was assessed by combined immunoprecipitation and immunoblotting (Fig.3). Whereas overexpression of wild-type Cbl enhanced ligand-dependent EGF-R ubiquitination (Fig. 3, compare lanes 2 and 4), ubiquitination was suppressed by the transforming deletion mutant proteins Cbl-70Z, Δ368 and Δ371 (Fig. 3, compare lane 2 with lanes 6, 12, and 14, respectively).

Each deletion mutant protein was able to associate with EGF-R (Bowtell and Langdon, 1995 and data not shown), yet failed to effect enhanced receptor down-regulation and ubiquitination. Thus, the transformation potential of Cbl linker region deletion mutants correlates with their dominant negative behavior in these assays.

Tyrosine at Position 371 Is Critical for Cbl-mediated Enhancement of EGF-R Down-regulation and Ubiquitination, But Tyrosine at Position 368 Is Not-- The contribution of Cbl amino acids Y368 and Y371 to EGF-R regulation was further investigated using amino acid substitution mutants targeting these residues. A panel of mutants was generated for the individual

substitution of phenylalanine (F), glutamic acid (E), or alanine (A) for each tyrosine (see Table I). Substitutions were chosen for one of the following reasons: 1) to conserve structure while preventing phosphorylation of the target residue (Y-to-F); 2) to alter structure while conserving the charge that would result from tyrosine phosphorylation (Y-to-E); 3) to alter both structure and potential charge (Y-to-A).

The activity of the substitution mutants was evaluated in the EGF-R down-regulation assay. All three Y371 substitution mutants functioned as dominant-negative regulators of surface EGF-R down-regulation (Fig.4, open symbols/solid lines), thereby mimicking the activity of the dominant-negative Δ371 Cbl mutant (Fig.2, -X-). However, Y368 substitution mutants were as active as wild-type Cbl in enhancing EGF-R down-regulation (Fig.4, compare squares with open symbols/dashed lines). These results contrasted with the dominant-negative function of mutant Δ368 in this assay (Fig.2, triangles).

The impact of the Y368 and Y371 substitution mutants on EGF-R ubiquitination was determined. Each Cbl mutant was able to associate with the EGF-R (data not shown). While the Y368 substitution mutants enhanced EGF-R ubiquitination to the same degree as did wild-type Cbl (Fig.5 middle panel, compare lanes 4, 6, 8, and 10 with lane 2), the Y371 substitution mutants effected no enhancement (Fig.5 middle panel, compare lanes 12, 14, and 16 with lane 2). Control immunoblots of cell lysates confirmed that EGF-R (Fig.5, top panel) and wild-type and mutant Cbl proteins were expressed at equivalent levels in all transfections, with the exception of the Y371A mutant protein (Fig.5, bottom panel). Immunofluorescence microscopy of cells expressing the Y371A mutant protein revealed accumulation of GFP-tagged Cbl in cytoplasmic aggregates, rather than the cytosolic distribution observed for wild-type or other mutant Cbl proteins (not shown). Aberrant intracellular localization correlated with an apparent reduction

of protein solubility in the lysis buffer used in the combined immunoprecipitation and immunoblotting analysis (Fig.5 bottom panel, lanes 15 and 16).

Taken together, our results indicate that a tyrosine at position 371 of Cbl is indispensable for normal regulation of the EGF-R, while the identity of the residue at position 368 is not critical. Instead, amino acid 368 serves as a critical spacer residue within the Cbl linker region. The implications of these findings are addressed in the Discussion.

Specific Amino Acid Substitutions of Cbl Residues T364, S376, and T377 Compromise Cbl-mediated Enhancement of EGF-R Down-regulation and Ubiquitination-- In addition to Y368 and Y371, three other linker region amino acids (T364, S376, and T377) could potentially undergo ligand-dependent phosphorylation. Using expression constructs encoding singly substituted Cbl proteins (see Table I), we assessed the contribution of these amino acids to Cbl-mediated enhancement of EGF-R down-regulation and ubiquitination. Substitutions were designed to effect either of two changes: 1) to mimic the charge that would be induced upon phosphorylation of the residue without conserving amino acid structure (S- or T-to-D); or 2) to conserve neither amino acid structure nor potential charge (S- or T-to-A).

The results of the EGF-R down-regulation assays using the linker serine and threonine substitution mutants are shown in Figure 6. Mutations at position 364 gave varied results. While the T364D mutant functioned like wild-type Cbl in enhancing EGF-R down-regulation (Fig.6A, -X-), Cbl T364A was compromised for this activity (Fig.6A, triangles). In several ways, the GFP-T364A mutant resembled the Y371A mutant: it localized to cytoplasmic aggregates (data not shown) and was poorly soluble in the lysis buffer used to prepare cell lysates (Fig.7, bottom

panel, lanes 4 and 12). However, the dominant-negative activity of the Y371A mutant was not reflected by the T364A mutant, which slightly enhanced EGF-R down-regulation in this assay.

Substitution of alanine for serine at position 376 had some impact in the down-regulation assay. The mutant enhanced EGF-R down-regulation, but to a lesser degree than did wild-type Cbl (Fig.6B, compare triangles and squares).

The Cbl T377D mutant was functionally null for enhancement of EGF-R down-regulation (Fig.6C, -X-). However, the T377A mutant enhanced down-regulation to near wild-type levels (Fig.6C, triangles).

These data show that at least one amino acid substitution at any of the three linker region serine or threonine residues can reduce or abrogate Cbl-mediated enhancement of EGF-R down-regulation. To confirm that these mutations similarly compromise EGF-R ubiquitination, we tested the mutants in the combined immunoprecipitation and immunoblotting procedure (Fig.7). All Cbl mutants that enhanced EGF-R down-regulation also enhanced ligand-dependent receptor ubiquitination (Fig.7, middle panel, compare lanes 10, 11, 12, and 14 with lane 8). These included mutants T364D, T364A, S376A, and T377A. The T377D mutant, unable to enhance EGF-R down-regulation, was badly compromised in its ability to enhance receptor ubiquitination (Fig.7, middle panel, compare lane 13 with lane 8).

In summary, results from the linker region mutant analyses suggest that the Cbl activities effecting enhanced ligand-dependent EGF-R down-regulation and enhanced ligand-dependent receptor ubiquitination are linker-dependent and genetically inseparable.

Conclusions

Cbl-mediated enhancement of EGF-R ubiquitination, down-regulation, and degradation is ligand-dependent. The Cbl RING finger plays a critical role in these activities. Waterman and colleagues showed that the substitution of a single cyteine within the RING finger abrogates Cbl-mediated enhancement of EGF-R down-regulation, ubiquitination, and degradation (18). Very recently, two groups reported that E2 proteins of the ubiquitin conjugation pathway can cooperate with Cbl to effect EGF-R ubiquitination (19, 20). Both groups demonstrated that an intact Cbl RING finger domain is required for this activity. Levkowitz and colleagues found that the E2 proteins Ubc-H5B and Ubc-H5C, but not Ubc-H7, could enhance EGF-R ubiquitination in an *in vitro* ubiquitin conjugation system (20). Their results differ from those of Yokouchi and coworkers, who showed that Ubc-H7 but not Ubc-H5 could associate with the Cbl RING-containing sequences, and in conjunction with Cbl could enhance EGF-R ubiquitination *in vitro* and *in vivo* (19). In these experiments, Cbl amino acids 374-430 were sufficient to mediate interaction with Ubc-H7 in a yeast two-hybrid screen. However, Cbl sequences N- and C-terminal to this region were required for *in vitro* association of Cbl and Ubc-H7. Notably, the required N-terminal sequences include the Cbl linker region. This suggests that the linker region regulates ligand-dependent recruitment of E2 proteins to the Cbl RING finger *in vivo*.

We demonstrate here that the Cbl linker region contains multiple residues that contribute to Cbl-mediated enhancement of EGF-R down-regulation and ubiquitination. The integrity of Cbl tyrosine 371 is crucial for enhanced receptor ubiquitination (20). We find that Y371 is critical for both ubiquitination and down-regulation of the EGF-R. Linker amino acids T364, S376, and T377 also contribute to Cbl-mediated EGF-R down-regulation and ubiquitination.

A striking finding presented here concerns the role of linker residue Y368 in effecting negative regulation of the EGF receptor. While substitution of this residue has no consequence in assays of EGF-R down-regulation and ubiquitination, its deletion converts Cbl into a dominant-negative protein (see Figs.2-3) with the potential to transform mammalian cells (42). These results suggest that Y368 functions within the linker region as a spacer residue. The obligatory conclusion to be drawn from this is that the linker region comprises a domain whose structure regulates Cbl function.

Although a mechanism of action for the Cbl linker region has not been defined, at least two models can be proposed. In the first, the linker region would undergo EGF-dependent phosphorylation on one or more of the five linker tyrosine, serine, and threonine residues. Linker phosphorylation would induce local changes in secondary structure, affecting the adjacent RING finger domain and rendering it competent to bind E2 proteins. In the second model, EGF-induced structural changes in the linker region would create a binding site within the linker for recruitment of additional ubiquitin conjugation pathway proteins. Both models invoke an activation-induced change in linker region secondary structure.

Specific secondary structure can mark proteins for degradation. Putative α-helices are critical for the proteasome-dependent degradation of several targets (43, 44). Furthermore, amphipathic α-helices within substrates can target them for ubiquitin-dependent proteolysis. In an analysis of synthetic signals mediating ubiquitin-dependent proteolysis, introduction of an amphipathic α-helix into a target protein sensitized it to degradation mediated collectively by the proteins Ubc6, Ubc7, and Ubc-4 or -5 (45). Two mammalian ubiquitin-conjugating proteins, Ubc-H7 and Ubc-H5, have been shown to cooperate with Cbl to enhance EGF-R ubiquitination *in vitro* (19, 20). Interestingly, predictions of protein secondary structure (46) suggest that the

Cbl linker region and other PEST-like sequences are enriched for α-helical content (not shown). It is possible that ligand-dependent phosphorylation induces the linker region to adopt an α-helical conformation, rendering it competent to recruit Ubc proteins to Cbl.

Further experiments will be required to investigate these possibilities. Although genetic analyses suggest that the Cbl linker region undergoes inducible phosphorylation and that such modification is critical for Cbl-mediated regulation of the EGF-20, 42, and this study), phosphorylation of any residue within the linker region has yet to be demonstrated. Based on our results, we conclude that multiple residues within the Cbl linker region contribute to Cbl-enhanced down-regulation and ubiquitination of the EGF-R, and that the structure of this domain is crucial to those functions.

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1 The abbreviations used are: EGF-R, epidermal growth factor receptor; TKB, tyrosine kinase-binding; GFP, green fluorescent protein; HEK, human embryonic kidney; EGF, epidermal growth factor; FBS, fetal bovine serum; MFI, mean fluorescence intensity.

Figure Legends

FIG. 1. **Structural domains of mammalian c-Cbl, *C. elegans* SLI-1, and Drosophila D-Cbl.** The structures of the evolutionarily conserved proteins are aligned. The c-Cbl sequences sufficient to mediate enhanced EGF-R down-regulation and ubiquitination lie within amino acids 1-440. This region encompasses the receptor-binding TKB domain, the linker region, the RING finger domain (RF) and a short stretch of C-terminal amino acids. The amino acid sequence of the c-Cbl linker region is shown at the bottom. Potential phosphorylation sites within the linker region are marked by asterisks.

FIG. 2. **The transforming Cbl deletion mutants 70Z, Δ368, and Δ371 suppress down-regulation of EGF-R from the cell surface.** Replicate cultures of 293 HEK cells in 10 cm dishes were transiently transfected using the EGF-R/pAlterMAX construct (0.1 μg), in combination with the indicated GFP-Cbl constructs (4 μg). Total DNA was 4.1 μg per dish. At 48 hr post-transfection, cultures were subjected to serum starvation followed by incubation with EGF (100 ng/ml) for the indicated times at 37°C. Samples were processed for live cell immunostaining and flow cytometry as detailed in Materials and Methods. The EGF-R-specific MFI at each timepoint is expressed as a percentage of the initial EGF-R-specific MFI of unstimulated cells that were identically transfected. The data shown are representative of the curves obtained in three independent experiments. Symbols: GFP, diamonds; GFP-Cbl-wt, squares; GFP-Cbl-70Z, circles; GFP-Cbl-Δ368, triangles; GFP-Cbl-Δ371, -X-.

FIG. 3. **The transforming Cbl deletion mutants 70Z, Δ368, and Δ371 do not enhance EGF-R ubiquitination.** Cultures of 293 HEK cells in 10 cm dishes were transiently transfected using the EGF-R/pAlterMAX construct (0.1 μg), in combination with the indicated GFP-Cbl constructs (4 μg). Total DNA was 4.1 μg per dish. At 48 h post-transfection, cultures were subjected to serum starvation followed by incubation without (-) or with EGF (+) for 10 min at 37°C. Cell lysates were prepared as described in Materials and Methods. Equal amounts (1000 μg) of protein from each lysate were immunoprecipitated using anti-GFP antibody. Lysate proteins (100 μg) and immunoprecipitates were resolved by SDS-PAGE and transferred to a PVDF membranes. The membrane containing the immunoprecipitated proteins was sequentially immunoblotted using anti-ubiquitin and anti-EGF-R antibodies. The membrane containing the lysate samples was immunoblotted using anti-GFP antibody. The position of the 175 kDa prestained molecular mass marker is shown to the right of the immunoprecipitation panels.

FIG. 4. **The Y368 substitution mutants down-regulate EGF-R from the cell surface as efficiently as does wild-type Cbl.** Replicate cultures of 293 HEK cells in 10 cm dishes were transiently transfected using the EGF-R/pAlterMAX construct (0.1 μg), in combination with the indicated GFP-Cbl constructs (4 μg). Total DNA was 4.1 μg per dish. At 48 hr post-transfection, cultures were subjected to serum starvation followed by incubation with EGF (100 ng/ml) for the indicated times at 37°C. Samples were processed for live cell immunostaining and flow cytometry as detailed in Materials and Methods. The EGF-R-specific MFI at each timepoint is expressed as a percentage of the initial EGF-R-specific MFI of unstimulated cells that were identically transfected. The data shown are representative of the curves obtained in

three independent experiments. Symbols: GFP, diamonds; GFP-Cbl-wt, squares; GFP-Cbl-70Z, closed triangles; GFP-Cbl-Y368F, -X-/dashed line; GFP-Cbl-Y368E, open triangles/dashed line; GFP-Cbl-Y368A, open circles/dashed line; GFP-Cbl-Y371F, -X-/solid line; GFP-Cbl-Y371E, open triangles/solid line; GFP-Cbl-Y371A, open circles/solid line.

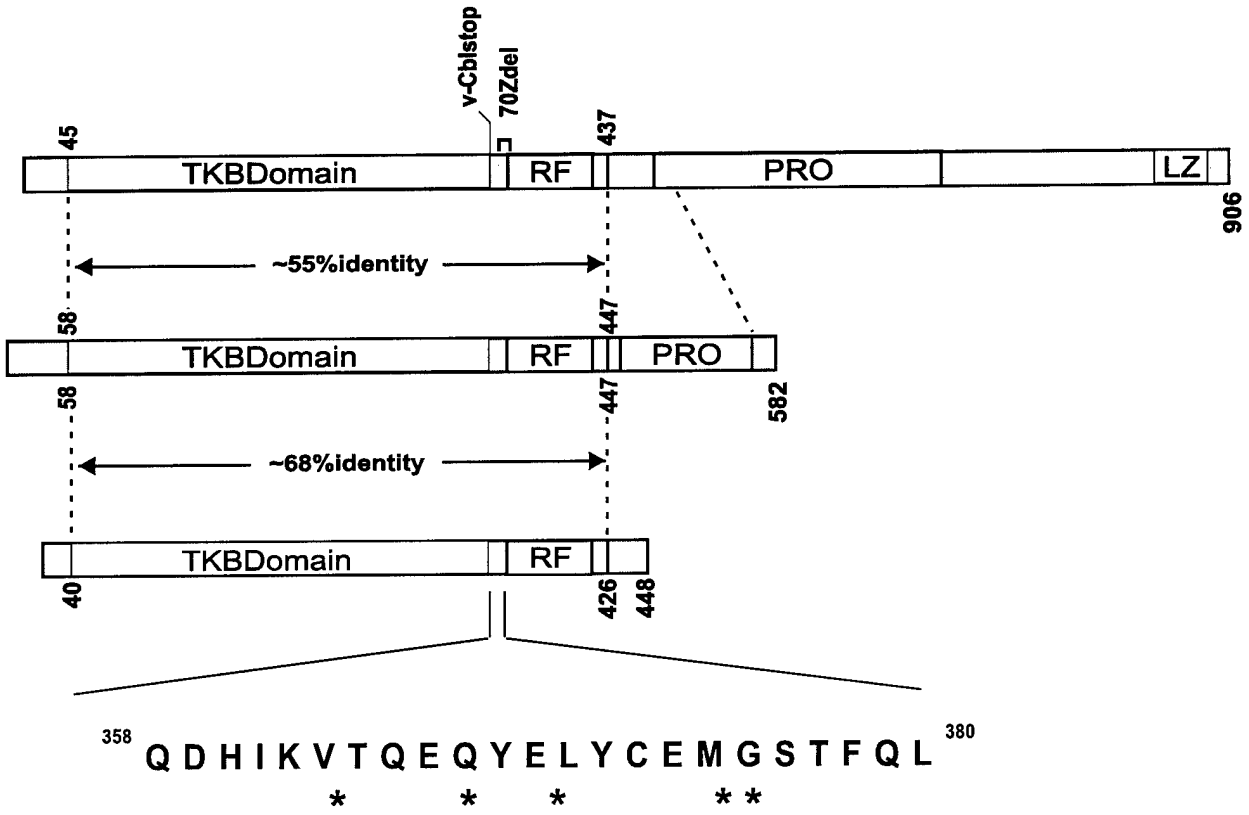
FIG. 5. **The Y368 substitution mutants enhance EGF-R ubiquitination, but the Y371 substitution mutants do not.** Cultures of 293 HEK cells in 10 cm dishes were transiently transfected using the EGF-R/pAlterMAX construct (0.2 μg), in combination with the indicated GFP-Cbl constructs (2 μg). Total DNA was 2.2 μg per dish. At 48 h post-transfection, cultures were subjected to serum starvation followed by incubation without (-) or with EGF (+) for 10 min at 37°C. Cell lysates were prepared as described in Materials and Methods. Equal amounts (900 μg) of protein from each lysate were immunoprecipitated using anti-GFP antibody. Lysate proteins (90 μg) and immunoprecipitates were resolved by SDS-PAGE and transferred to a PVDF membranes. The membrane containing the immunoprecipitated proteins was sequentially immunoblotted using anti-ubiquitin and anti-EGF-R antibodies. The membrane containing the lysate samples was immunoblotted using anti-GFP antibody. The position of the 175 kDa prestained molecular mass marker is shown to the right of the immunoprecipitation panels.

FIG. 6. **The Cbl linker mutants substituted at residue 364, 376, or 377 have variable impact on down-regulation of EGF-R from the cell surface.** Replicate cultures of 293 HEK cells in 10 cm dishes were transiently transfected using the EGF-R/pAlterMAX construct (0.1 μg), in combination with the indicated GFP-Cbl constructs (4 μg). Total DNA was 4.1 μg per dish. At 48 hr post-transfection, cultures were subjected to serum starvation followed by incubation with

EGF (100 ng/ml) for the indicated times at 37°C. Samples were processed for live cell immunostaining and flow cytometry as detailed in Materials and Methods. The EGF-R-specific MFI at each timepoint is expressed as a percentage of the initial EGF-R-specific MFI of unstimulated cells that were identically transfected. The data shown are representative of the curves obtained in three independent experiments. **A)** T364 substitution mutants. **B)** S376 substitution mutant. **C)** T377 substitution mutants. Symbols: GFP, closed diamonds; GFP-Cbl-wt, closed squares; GFP-Cbl-70Z, closed circles; substitution of D for wild-type residue, -X-; substitution of A for wild-type residue, open triangles.

FIG. 7. **The Cbl linker mutants substituted at residue 364, 376, or 377 have variable impact on EGF-R ubiquitination.** Cultures of 293 HEK cells in 10 cm dishes were transiently transfected using the EGF-R/pAlterMAX construct (0.1 μg), in combination with the indicated GFP-Cbl constructs (4 μg). Total DNA was 4.1 μg per dish. At 48 h post-transfection, cultures were subjected to serum starvation followed by incubation without (-) or with EGF (+) for 10 min at 37°C. Cell lysates were prepared as described in Materials and Methods. Equal amounts (800 μg) of protein from each lysate were immunoprecipitated using anti-GFP antibody. Lysate proteins (80 μg) and immunoprecipitates were resolved by SDS-PAGE and transferred to a PVDF membranes. The membrane containing the immunoprecipitated proteins was sequentially immunoblotted using anti-ubiquitin and anti-EGF-R antibodies. The membrane containing the lysate samples was immunoblotted using anti-GFP antibody. The position of the 175 kDa prestained molecular mass marker is shown to the right of the immunoprecipitation panels.

Fig. 1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PRO
LZ
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~68%identity
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358 Q D H I K V T Q E Q Y E L Y C E M G S T F Q L 380
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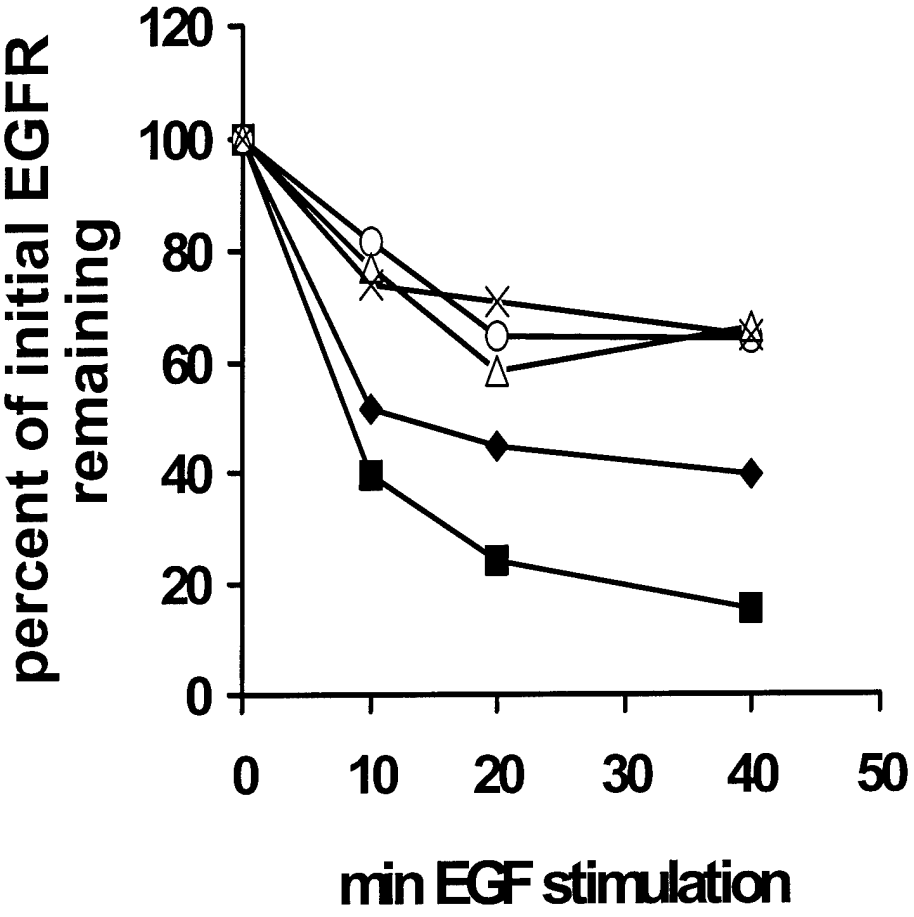
Fig. 2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40
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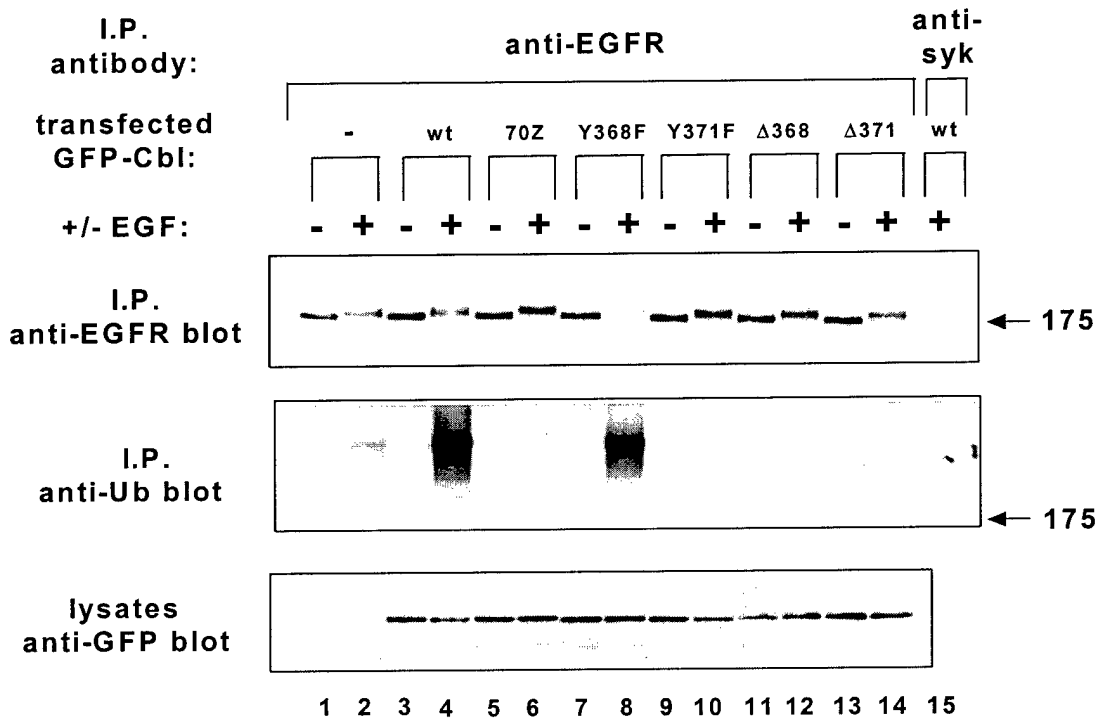
0
0
10
20
30
40
50
min EGF stimulation

Fig. 3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anti-EGFR
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-
wt
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Y371F
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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Fig. 4

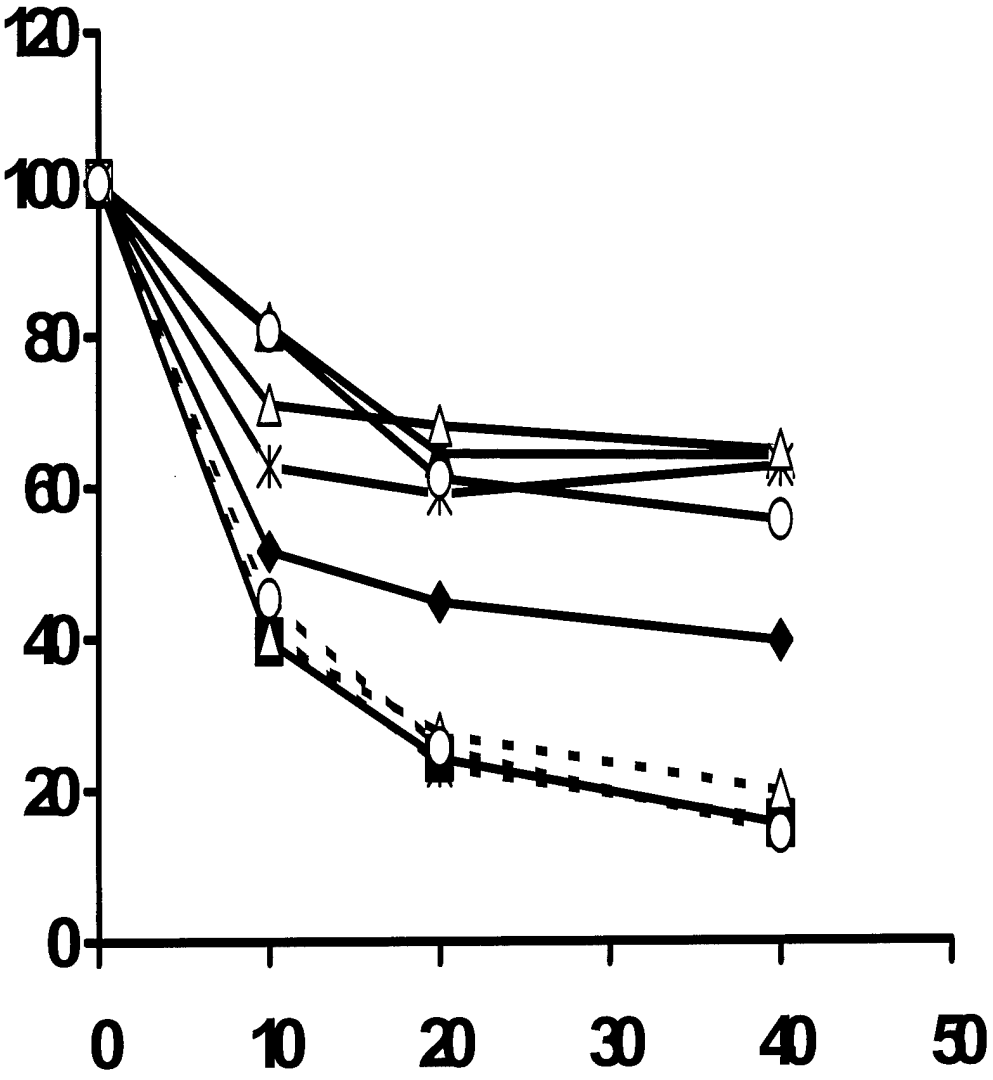

Fig. 5

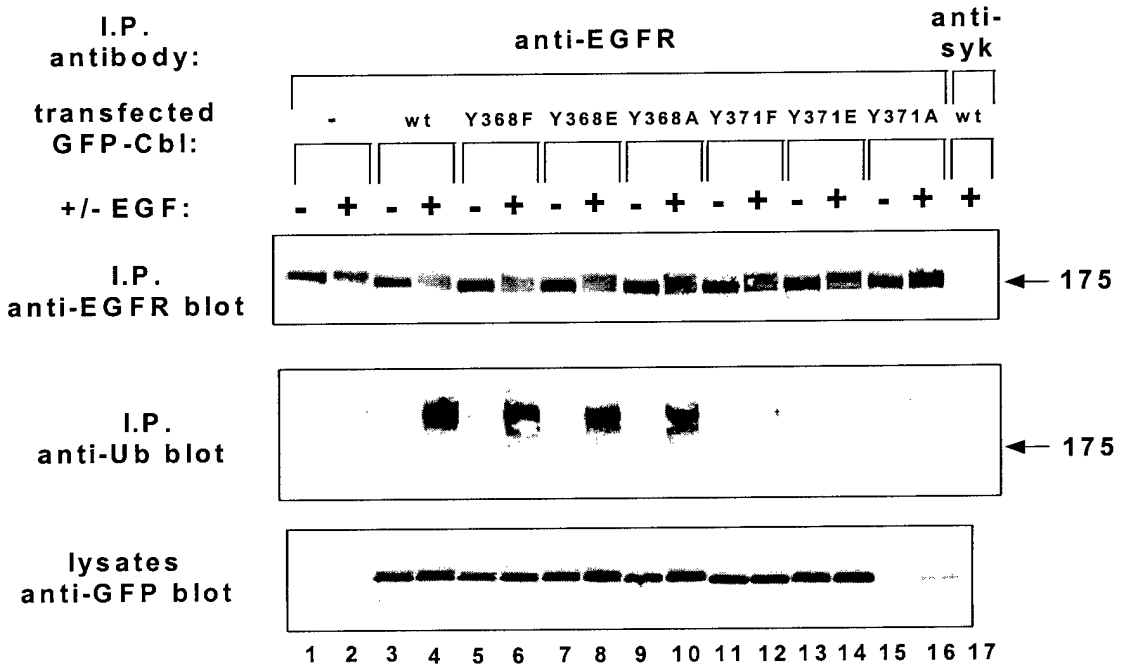
I.P. antibody:
anti-EGFR
anti-syk
transfected GFP-Cbl:
-
wt
Y368F
Y368E
Y368A
Y371F
Y371E
Y371A
wt
+/- EGF:
- + - + - + - + - + - + - + - + +
I.P. anti-EGFR blot
175
I.P. anti-Ub blot
175
lysates anti-GFP blot
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Fig. 6

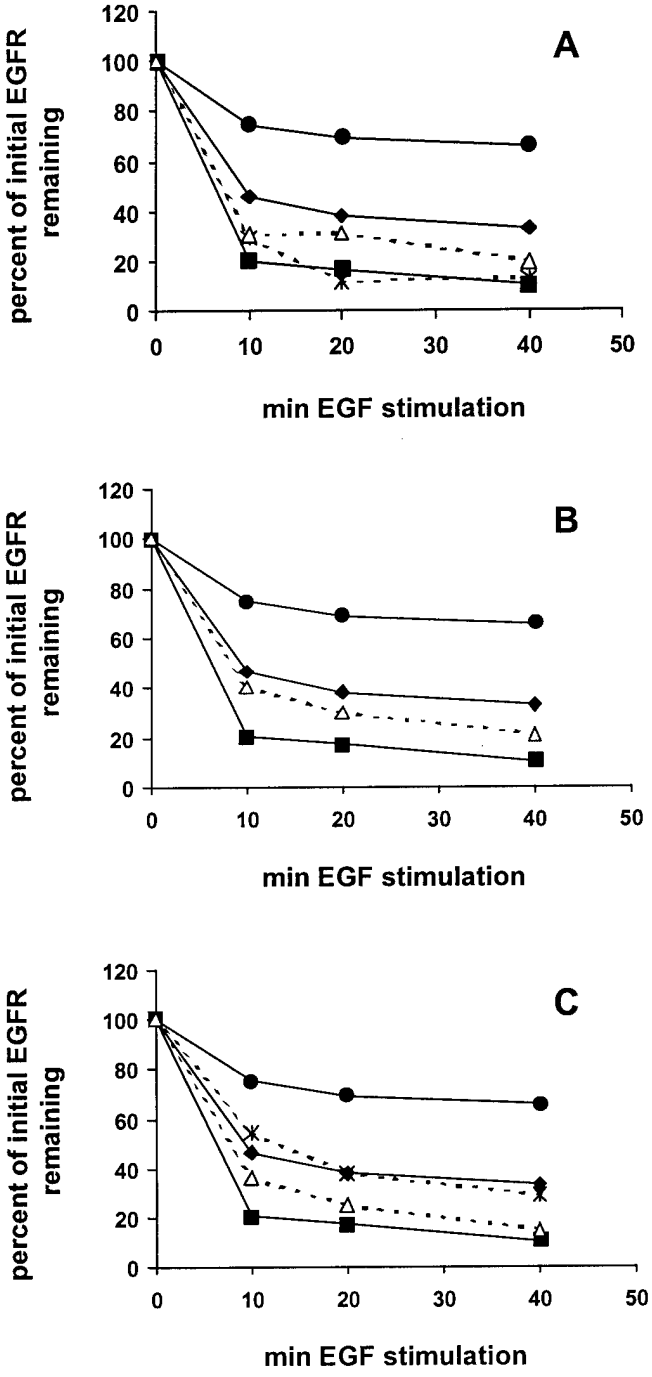
A
B
C
percent of initial EGFR remaining
120
100
80
60
40
20
0
0
10
20
30
40
50
min EGF stimulation

Fig. 7

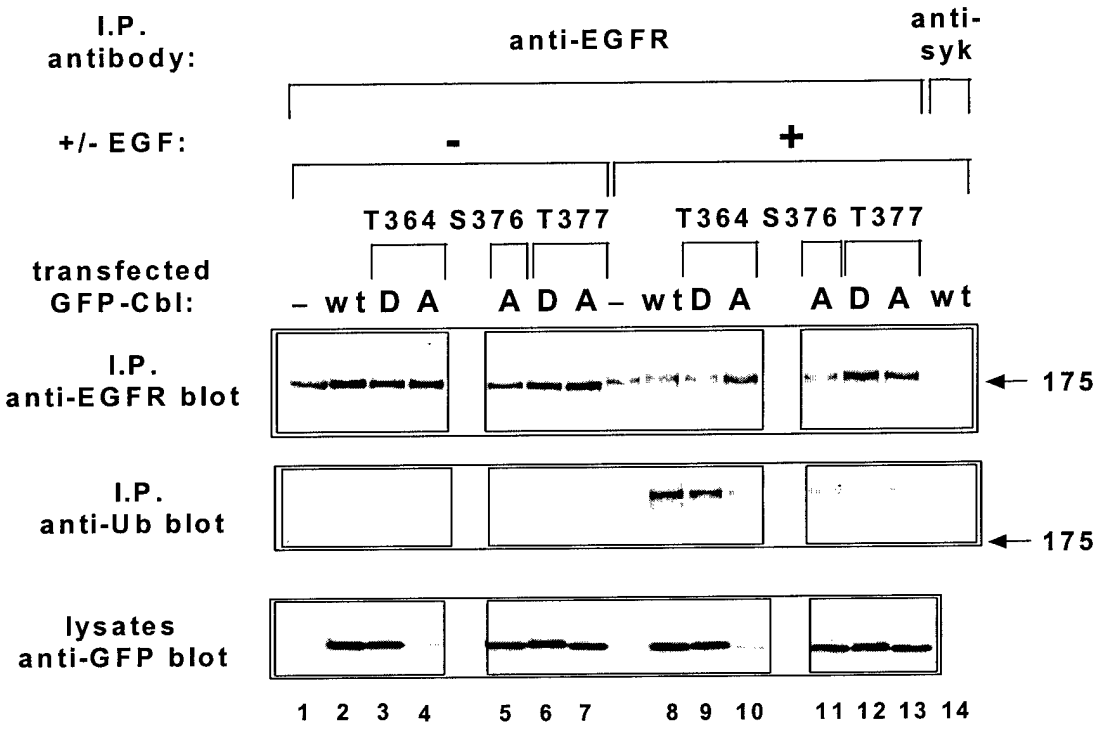
I.P. antibody:
anti-EGFR
anti-syk
+/- EGF:
-
+
T364 S376 T377
T364 S376 T377
transfected GFP-Cbl:
– wt D A
A D A – wt D A
A D A wt
I.P. anti-EGFR blot
175
I.P. anti-Ub blot
175
lysates anti-GFP blot
1 2 3 4
5 6 7
8 9 10
11 12 13 14

TABLE I. List of Cbl Linker Region Substitution Mutants Generated for This Study

Mutation	Mutagenic Oligonucleotide	Construct Name
Y368F	5'-ACA-GTA-TAA-TTC-AAA-TTG-TTC-CTG-G-3'	GFP-Y368F
Y368E	5'-ACA-GTA-TAA-TTC-CTC-TTG-TTC-CTG-G-3'	GFP-Y368E
Y368A	5'-ACA-GTA-TAA-TTC-AGC-TTG-TTC-CTG-G-3'	GFP-Y368A
Y371F	5'-GCC-CAT-CTC-ACA-GAA-TAA-TTC-ATA-TTG-3'	GFP-Y371F
Y371E	5'-GCC-CAT-CTC-ACA-CTC-TAA-TTC-ATA-TTG-3'	GFP-Y371E
Y371A	5'-GCC-CAT-CTC-ACA-GGC-TAA-TTC-ATA-TTG-3'	GFP-Y371A
T364D	5'-ATA-TTG-TTC-CTG-GTC-CAC-TTT-GAT-ATG-3'	GFP-T364D
T364A	5'-ATA-TTG-TTC-CTG-GGC-CAC-TTT-GAT-ATG-3'	GFP-T364A
S376A	5'-GTT-GGA-ATG-TGG-CGC-CCA-TCT-CAC-3'	GFP-S376A
T377D	5'-CAT-AGT-TGG-AAG-TCG-GAG-CCC-ATC-3'	GFP-T377D
T377A	5'-CAT-AGT-TGG-AAT-GCG-GAG-CCC-ATC-3'	GFP-T377A